Identification of Three Species of *Borrelia burgdorferi Sensu Lato* (*B. burgdorferi Sensu Stricto*, *B. garinii*, and *B. afzelii*) Among Isolates from Acrodermatitis Chronica Atrophicans Lesions

Roger N. Picken,* Franc Strle,† Maria M. Picken,‡* Eva Ruzic-Sabljic,§ Vera Maraspin,† Stanka Lotric-Furlan,† and Joze Cimperman†

*Research Service, Hines Veterans' Administration Hospital, Maywood, Illinois, U.S.A.; †Department of Infectious Diseases, University Medical Center, Ljubljana, Slovenia; †Department of Pathology, Loyola University Medical Center, Maywood, Illinois, U.S.A.; §Institute of Microbiology, University of Ljubljana, Ljubljana, Slovenia

In Europe, at least three species of *Borrelia* are known to be causative agents of Lyme borreliosis: *B. burgdorferi sensu stricto, B. garinii*, and *B. afzelii*. Observable differences in the molecular characteristics of the three species have led to speculation that they may also differ in their pathogenic potential and/or tissue tropisms. Several studies have found an association between the chronic skin manifestation of Lyme borreliosis, acrodermatitis chronica atrophicans, and infection by *B. afzelii*. We sought to find further evidence for such a correlation by studying the genetic profiles of 22 strains of *B. burgdorferi sensu lato* derived from 21 patients who presented to the University Medical Center, Ljubljana, Slovenia between 1992 and 1995. Strains were isolated in culture from skin biopsies of acrodermatitis chronica atrophicans lesions; in the case of one patient two separate acrodermatitis chronica atrophicans lesions were cultured. All 21 patients had clinically typical lesions with "classic" histopathology and high IgG antibody titers to *B. burgdorferi sensu lato*. Strains were characterized and typed by 16S ribosomal RNA-specific polymerase chain reaction and determination of their large restriction fragment patterns using pulsed-field gel electrophoresis of *Mlu*I-digested genomic DNA. Of the 22 isolates studied, 17 possessed the highly conserved MLa1 pattern characteristic of *B. afzelii*. The remaining five isolates possessed large restriction fragment patterns that were typical of *B. garinii* (MLg2, four isolates from three patients) and *B. burgdorferi sensu stricto* (MLb2, one isolate). The results of 16S ribosomal RNA-specific polymerase chain reaction were concordant with these species designations. These data show that *B. afzelii* is the predominant, but not the exclusive, etiologic agent of acrodermatitis chronica atrophicans. *Key words: molecular characterization/patient isolates. J Invest Dermatol 110:211–214, 1998*

Acrodermatitis chronica atrophicans (ACA) is a late cutaneous manifestation of Lyme borreliosis characterized by erythrocyanotic lesions that typically involve acral sites (Åsbrink and Hovmark, 1987, 1988; Abele and Anders, 1990). Untreated disease has a long progressive course that comprises both inflammatory and atrophic stages where the evolution of lesions is slow and insidious. In the atrophic phase, generalized thinning of the dermis and epidermis gives the skin a transparent appearance, often with clearly visible veins (Abele and Anders, 1990; Åsbrink, 1993). Unlike other skin manifestations, such as erythema migrans and borrelial lymphocytoma, ACA does not heal without treatment; the inflammatory stage may last for months or years and the atrophic stage can endure for decades (Åsbrink, 1993). The borrelial etiology of the disease was firmly established in 1984 by the cultivation of *Borrelia burgdorferi* from an ACA lesion (Åsbrink *et al*, 1984).

Within the general taxon *B. burgdorferi sensu lato*, three species are known to be causative agents of human Lyme borreliosis: *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii* (Baranton *et al*, 1992). There is disparity in their geographic distribution, however; whereas all three species have been encountered among European patient isolates, only *B. burgdorferi sensu stricto* has thus far been isolated from patients in North America. These findings have led to speculation that the unequal distribution of species might be associated with perceived differences in disease manifestations. For example, ACA has been widely encountered throughout Europe, but there have been few reports from the United States. Of 45 cases of ACA seen at the Mayo Clinic up to 1945, only six patients were born in the United States, and the remainder were immigrants, mostly from Europe (Montgomery and Sullivan, 1945). Since then, only a few additional reports from North America have appeared (Steere *et al*, 1986; Lavoie *et al*, 1986; Tuffanelli, 1987; Kaufman *et al*, 1989; Edwards *et al*, 1992).

Such observations have led to a search for associations between individual *Borrelia* spp. and the clinical manifestations of Lyme borreliosis. In the case of ACA, indications of just such a correlation were found. Molecular studies of ACA isolates or sera from several European countries over the last 4 y have produced evidence suggesting an exclusive association of *B. afzelii* with ACA (van Dam *et al*, 1993; Assous *et al*, 1993; Anthonissen *et al*, 1994; Balmelli and Piffaretti, 1995; Wienecke *et al*, 1995; Ohlenbusch *et al*, 1996). To further investigate this phenomenon, we examined 22 new isolates of *B. burg-*

Manuscript received October 29, 1996; revised November 11, 1997; accepted for publication November 19, 1997.

Reprint requests to: Dr. Maria M. Picken, Department of Pathology, Room 2242, Building 110, Loyola University Medical Center, 2160 South First Avenue, Maywood, Illinois 60153.

Abbreviations: ACA, acrodermatitis chronica atrophicans; CSF, cerebrospinal fluid; LRFP, large restriction fragment pattern; rRNA, ribosomal RNA.

Table I. *Borrelia* spp. and strains used and results of molecular subtyping studies

Species	Strain designation	LRFP[a]	Biologic origin	Geographic location
Reference strains				
DN127 genomic group	DN127	U	Tick (*Ixodes pacificus*)	U.S.A.
DN127 genomic group	25015	U	Tick (*Ixodes scapularis*)	U.S.A.
B. burgdorferi sensu stricto	B31 [ATCC 35210]	MLb1	Tick (*Ixodes scapularis*)	U.S.A.
B. burgdorferi sensu stricto	297 [ATCC 53899]	MLb2	CSF[b]	U.S.A.
B. garinii	20047	MLg1	Tick (*Ixodes ricinus*)	France
B. garinii	PBi	MLg2	CSF	Germany
B. afzelii	VS461	MLa1	Tick (*Ixodes ricinus*)	Switzerland
VS116 genomic group	VS116	U	Tick (*Ixodes ricinus*)	Switzerland
Strains from patients				
B. burgdorferi sensu stricto	SL-91	MLb2	Skin (ACA[c])	Slovenia
B. garinii	SL-92	MLg2	Skin (ACA)	Slovenia
B. garinii	SL-95	MLg2	Skin (ACA)	Slovenia
B. garinii	SL-109	MLg2	Skin (ACA)	Slovenia
B. afzelii	SL-94	MLa1	Skin (ACA)	Slovenia
B. afzelii	SL-112	MLa1	Skin (ACA)	Slovenia

[a]LRFP, large restriction fragment pattern. Nomenclature follows the system devised previously (Belfaiza *et al*, 1993) for bands >70 kb in size. Thus, all LRFP derived from restriction endonuclease *Mlu* I digestion are designated ML, *B. burgdorferi sensu stricto* LRFP are designated MLb, *B. garinii* LRFP are designated MLg, and *B. afzelii* LRFP are designated MLa. U, undesignated LRFP.
[b]CSF, cerebrospinal fluid.
[c]ACA, acrodermatitis chronica atrophicans.

dorferi sensu lato derived from Slovene patients with ACA lesions. This report presents data on the molecular subtyping of these strains.

MATERIALS AND METHODS

Patients All patients presented to the University Medical Center, Lubljana, between July 1992 and June 1995. Criteria for the inclusion of patients in the study were as follows: (i) they had not received antibiotic treatment specifically for ACA, (ii) they had typical lesions on the extremities, (iii) histopathologic findings supported the diagnosis of ACA, (iv) IgG antibody titers against *B. burgdorferi sensu lato* were detectable, and (v) *B. burgdorferi sensu lato* was isolated in culture from a skin biopsy.

Skin biopsies and cultures Skin biopsies were taken from areas where the ACA lesions were most prominent. In the case of one patient two separate ACA lesions were cultured. Methods pertaining to the excision of tissue, the inoculation into growth medium, and the isolation of strains have been described previously (Strle *et al*, 1995a). In some patients lumbar puncture was also performed and cerebrospinal fluid (CSF) cultured for *B. burgdorferi sensu lato*. Standard microbiologic techniques were employed to preclude the possibility of cross-contamination of cultures. Representative *B. burgdorferi sensu lato* isolates obtained from ACA lesions of patients are listed in **Table I** along with reference strains used in comparative studies. All patient strains used in this study were low passage isolates.

Antibodies to *B. burgdorferi sensu lato* For all patients IgM and IgG antibody titers against *B. burgdorferi sensu lato* were determined on the day of skin biopsy by immunofluorescence assay (Wilske *et al*, 1984). A local isolate of *B. afzelii* was used as antigen. Titers of 1:256 or higher were interpreted as positive.

Species identification Isolates were identified to the species level using two independent methods: (i) 16S ribosomal RNA (rRNA)-specific polymerase chain reaction (PCR), (ii) large restriction fragment pattern (LRFP) analysis involving cleavage of genomic DNA with the restriction enzyme *Mlu*I and separation of the fragments using pulsed-field gel electrophoresis. Well-characterized reference strains of *B. burgdorferi sensu lato* were included for comparison (**Table I**). The strains B31, 20047, and VS461 are the type strains of the species *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii*, respectively. Isolates representing other genomic groups of *B. burgdorferi sensu lato* included strains DN127 and 25015 (DN127 group), and VS116 (VS116 group). The LRFP produced by restriction enzyme digestion are characteristic of each species (Belfaiza *et al*, 1993). Both techniques were performed as described previously (Strle *et al*, 1995a,b; Picken *et al*, 1995, 1996).

RESULTS

Patients From July 1992 to June 1995, 104 Slovene patients presenting with ACA were biopsied and cultured for *B. burgdorferi sensu lato*. In one of the patients two separate lesions were biopsied. Twenty-four lesions from 23 patients grew spirochetes, for an overall isolation rate of 22%. In addition, spirochetes were concomitantly isolated from the CSF of one patient. Twenty-two ACA isolates from 21 patients were subsequently studied by molecular subtyping methods. The patients comprised 14 females (67%) and seven males (33%), aged 21–76 y (median 66 y). The duration of ACA lesions varied from 5 mo to more than 20 y (median 2 y). Eighteen of the 21 patients recalled one or more tick bites in the preceding years and seven patients had a prior recollection of erythema migrans, which occurred on the same extremity as the subsequent ACA lesion in five patients. By immunofluorescence assay, all 21 patients had an IgG antibody response to *B. burgdorferi sensu lato*, and four also demonstrated an IgM response.

Table II. Frequency of isolation of *Borrelia* spp. from acrodermatitis chronica atrophicans lesions

Species	LRFP	No. of isolates obtained
B. burgdorferi sensu stricto	MLb2	1
B. garinii	MLg2	4[a]
B. afzelii	MLa1	17

[a]Two isolates were obtained from separate ACA lesions of one patient.

16S rRNA-specific PCR PCR analysis of the 22 isolates indicated that 17 isolates were *B. afzelii*, four were *B. garinii*, and one was *B. burgdorferi sensu stricto* (**Table II**). In one patient, where isolates were obtained from two separate ACA lesions, both strains typed as *B. garinii*. **Figure 1** shows the results of PCR amplification of DNA from six patient ACA isolates: *B. burgdorferi sensu stricto* strain SL-91, *B. garinii* strains SL-92, SL-95, and SL-109, and two representatives (SL-94, SL-112) from the 17 *B. afzelii* isolates. Only one of the two *B. garinii* isolates obtained from the same patient is shown (SL-95). Eight well-characterized reference strains, representing the DN127 genomic group, *B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, and the VS116 genomic group, are shown for comparison. Note, however, that the 16S rRNA-specific PCR primers are not able to differentiate *B. garinii* from the VS116 genomic group (**Fig 1**).

LRFP analysis **Figure 2** shows the results of LRFP analysis of the same six representative patient ACA isolates and the eight reference strains used in **Fig 1**. In all cases, results from the two methods of species identification were concordant. Seventeen of the 22 isolates typed as *B. afzelii*, possessing the same MLa1 LRFP as VS461, the *B. afzelii* type strain; this LRFP is characterized by fragments of ≈440, 320, and 90 kb (Picken *et al*, 1996). Four isolates typed as *B. garinii*, possessing the same MLg2 LRFP as the *B. garinii* reference strain PBi;

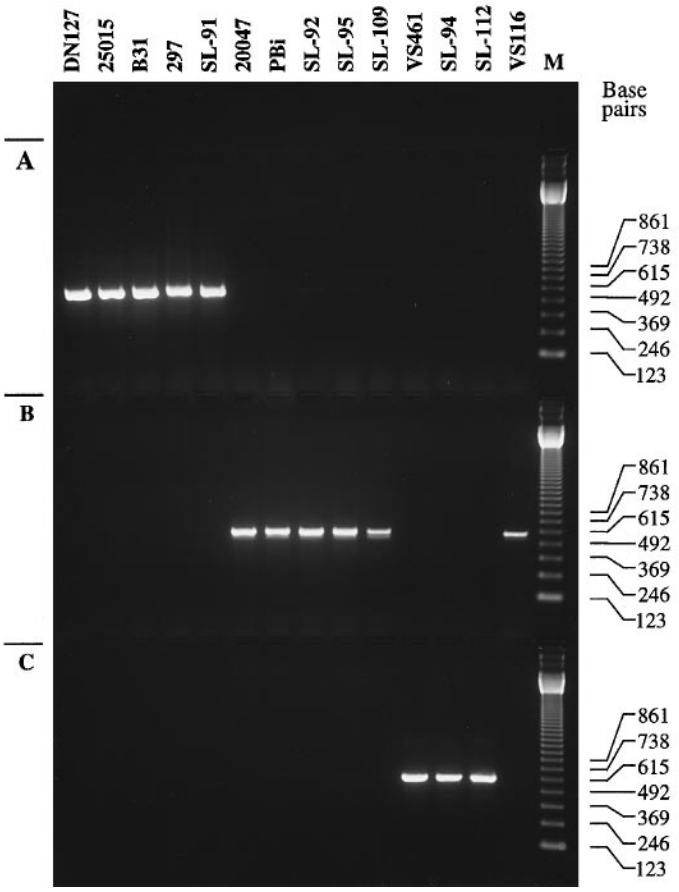

Figure 1. Molecular subtyping of patient ACA isolates by 16S rRNA-specific PCR. Genomic DNA from six representative ACA patient isolates and eight reference strains of *B. burgdorferi sensu lato* were amplified in PCR reactions using 16S rRNA primer sequences specific for *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii*. Amplification products were applied to an agarose gel with three sets of sample-loading wells, electrophoresed for 2.5 h, stained with ethidium bromide, and photographed using ultraviolet transillumination. Horizontal lines show the position of sample wells loaded with PCR products generated using: (*A*) *B. burgdorferi sensu stricto*-specific primers; (*B*) *B. garinii*-specific primers; (*C*) *B. afzelii*-specific primers. Strain designations are shown above their respective lanes (refer to **Table I** for the species designation of reference strains). *Lane M* contains molecular size markers (concatenates of a 123 bp fragment). Only two (SL-94, SL-112) of 17 isolates that typed as *B. afzelii* are shown, as representative examples.

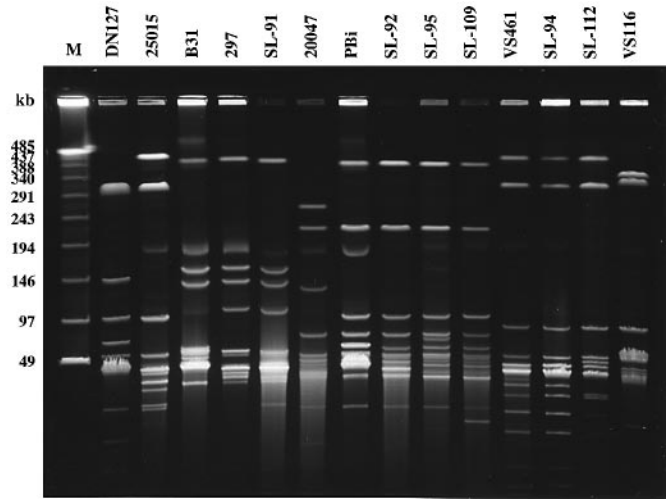

Figure 2. Molecular characterization of patient ACA isolates and reference strains by pulsed-field gel electrophoresis. Late logarithmic phase cells from the same six representative ACA patient isolates and eight reference strains shown in **Fig 1** were embedded in plugs of low melting temperature agarose and processed to lyse spirochetes. Slices from plugs were digested with restriction enzyme *Mlu*I and loaded into the slots of a pulsed-field gel electrophoresis gel. After electrophoresis for 24 h in a pulsed-field apparatus, the gel was stained with ethidium bromide and photographed using ultraviolet transillumination. Strain designations are shown above their respective lanes (refer to **Table I** for the species designation of reference strains). *Lane M* contains molecular size markers (λDNA concatamers of 48.5–485 kb). Only two (SL-94, SL-112) of 17 isolates that typed as *B. afzelii* are shown, as representative examples.

this LRFP is characterized by fragments of ≈390, 220, 100, and 80 kb (Picken *et al*, 1996). Three of the *B. garinii* patient isolates are shown in **Fig 2** (SL-92, SL-95, and SL-109). In one patient, where two isolates were obtained from separate ACA lesions, only one of the two strains is shown in **Fig 2** (SL-95), because both isolates produced identical macrorestriction patterns. One isolate (SL-91) typed as *B. burgdorferi sensu stricto*, possessing the same MLb2 LRFP as *B. burgdorferi sensu stricto* reference strain 297; this LRFP is characterized by fragments of ≈410, 160, 140, and 110 kb (Picken *et al*, 1996).

DISCUSSION

The 21 patients and 22 isolates described in this report represent the largest series of new, consecutive ACA patients and strains studied to date. Previously, the largest number of ACA patients investigated was represented by the 18 lesional skin biopsies studied by Wienecke *et al* (1995), from all of which *B. afzelii*-specific sequences were amplified by PCR. In contrast to their results, we found that four isolates typed as *B. garinii* and one isolate typed as *B. burgdorferi sensu stricto*. This is the second report of an association between *B. burgdorferi sensu stricto* and ACA; previously, the Danish isolate DK7, which was described as being derived from an ACA lesion, was typed as *B. burgdorferi sensu stricto* by an Outer Surface Protein A serotyping method (Wilske *et al*, 1993).

To date, only one, highly conserved LRFP (MLa1) has been described for *B. afzelii* (Belfaiza *et al*, 1993; Strle *et al*, 1995b; Picken *et al*, 1996). In this study, 17 of 22 isolates investigated possessed this MLa1 LRFP, demonstrating that they belong to the species *B. afzelii*. Six LRFP have been described for *B. garinii* (Belfaiza *et al*, 1993; Strle *et al*, 1995b; Picken *et al*, 1996), of which four have been encountered among Slovene patient isolates and six among Slovene tick isolates (Strle *et al*, 1995b; Picken *et al*, 1996). The four *B. garinii* strains isolated from ACA lesions in this study all possessed the MLg2 LRFP. This was also found to be the most prevalent *B. garinii* LRFP isolated from patients and ticks in Slovenia (Strle *et al*, 1995b; Picken *et al*, 1996) and may not therefore reflect a particular association with ACA. PBi, the MLg2 reference strain used in this study, was isolated from the CSF of a patient residing in Germany (Wilske *et al*, 1993). It would therefore seem that *B. garinii* of this type can be responsible for at least two of the sequelae of Lyme borreliosis, neuroborreliosis, and ACA.

Of the three species that infect humans, *B. burgdorferi sensu stricto* has been shown to be the most heterogeneous in terms of number of LRFP (Belfaiza *et al*, 1993; Strle *et al*, 1995b; Picken *et al*, 1996). The single *B. burgdorferi sensu stricto* isolate that we obtained from ACA possessed the MLb2 LRFP; previously, we have also encountered this LRFP among isolates derived from erythema migrans lesions of Slovene patients (Picken *et al*, 1996). *Borrelia burgdorferi sensu stricto* reference strain 297 (ATCC 53899), which also possesses the MLb2 LRFP, was isolated from the CSF of a North American patient. This suggests not only that strains of this type can be responsible for both neuroborreliosis and ACA, but also that the potential for ACA is present in North America.

The paucity of case reports from the United States has resulted in ACA being generally regarded as a "European" manifestation of chronic Lyme borreliosis; however, there have been sporadic reports of ACA from the United States over several years (Steere *et al*, 1986; Lavoie *et al*, 1986; Tuffanelli, 1987; Kaufman *et al*, 1989; Edwards *et al*, 1992). Recently, detailed case reports have been published of patients from New York who had clinically classic lesions and/or elevated serum

antibody titers to *B. burgdorferi* with demonstration of spirochetes by antibody staining (Kaufman *et al*, 1989; Edwards *et al*, 1992). The fact that *B. burgdorferi sensu stricto* has now been found associated with ACA lesions (Wilske *et al*, 1993; this study) supports the notion that cases of ACA can occur in the United States; however, reasons for its low incidence remain obscure.

Alternatively, it has also been shown that patients can be concomitantly infected with more than one *Borrelia* spp. (Demaerschalk *et al*, 1995). Thus, it is conceivable that the ACA lesions from which the *B. garinii* and *B. burgdorferi sensu stricto* isolates derived could actually have been caused by a concomitant *B. afzelii* infection, and that *in vitro* culture favored the outgrowth of *B. garinii* and *B. burgdorferi sensu stricto*. This possibility cannot be ruled out; however, it can be concluded that these latter species were present in the ACA skin lesion at the time of biopsy. Also, in the case where two isolates were obtained from separate ACA lesions of one patient, both strains had identical molecular properties (*B. garinii*, MLg2 subclass). In the case of another patient, isolates were obtained from both an ACA lesion and CSF; both strains typed as *B. afzelii* MLa1 (data not shown).

We conclude from these findings that in Slovene patients *B. afzelii* is not the sole etiologic agent of ACA and that this chronic skin manifestation can also result from infection with *B. garinii* and *B. burgdorferi sensu stricto*. The finding of involvement by the latter species suggests that the potential for ACA also exists in North America, and recent case reports from the United States appear to support this. It remains to be determined why a higher incidence of ACA is seen among Lyme borreliosis patients in Europe than in North America.

This work was supported in part by grant #AR 41517 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (to R.N.P.) and by a grant from the Schweppe Foundation (to M.M.P.). The authors are also grateful to Dr. Olivier Peter for supplying the reference strains VS461 and VS116.

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